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PRIMER NOTE

Isolation and characterization of polymorphic microsatellite loci in the boll weevil, *Anthonomus grandis* Boheman (Coleoptera: Curculionidae)

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Abstract

The boll weevil (*Anthonomus grandis* Boheman) is a major insect pest of cotton in North America. Dispersal activity poses a threat to ongoing eradication efforts in the US, but little is known about the frequency of long-distance migration. Nuclear molecular markers are needed to assess gene flow in relation to geographical distance. A biotin-enrichment strategy was employed to develop microsatellite markers for the boll weevil. Of 23 loci isolated, 14 were polymorphic with three to 10 alleles per locus. Twelve of the polymorphic loci showed Mendelian inheritance and are likely to be useful in population genetics studies.

Keywords: *Anthonomus grandis*, Boll weevil, microsatellites, molecular markers, population genetics

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The boll weevil (*Anthonomus grandis* Boheman) is a serious insect pest of cotton in the Western Hemisphere. This invasive insect has been eradicated from parts of the US Cotton Belt, and large-scale eradication programs are underway in areas still infested. An understanding of boll weevil dispersal patterns is crucial to evaluating the threat of migration into eradication zones. Recently, we utilized mtDNA PCR-RFLP and RAPD markers to characterize gene flow among boll weevil populations in the south central Cotton Belt of the US and northeast Mexico (Kim & Sappington 2004a,b). We hope further analyses based on microsatellite variation will help reconcile some conflicting results in the RFLP and RAPD analyses.

Microsatellite loci were isolated using a slight modification of biotin-enrichment methods (Kijas *et al*. 1994; Ronald *et al*. 2000). Fifty boll weevils were pooled and ground in liquid nitrogen. Stock DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega). Aliquots (30 μg) of the genomic DNA were fully digested with *Nde*II and electrophoresed in 1.5% low-melting-point (LMP) agarose in 1X TAE buffer. The 250–600 bp DNA fragments were excised, the LMP agarose was digested by AgarACE (Promega), and the fragments were purified using Microcon 100 spin-filters (Millipore). A double-stranded DNA linker with an *Nde*II compatible end was constructed as described by Ronald *et al*. (2000). The linker was incubated with the *Nde*II-digested DNA, and polymerase chain reaction (PCR) amplification was carried out in an Eppendorf thermocycler using the EP-3 primer of Ronald *et al*. (2000).

The biotinylated capture probe was annealed to the linkered DNA by combining 10 μL of linkered DNA, 1 μL (0.1 μg/μL) 5′-biotinylated capture probe [5′-biotin-$(CA)_{15}$, 5′-biotin-$(CT)_{15}$, 5′-biotin-$(GATA)_6$, HPLC-purified, Integrated DNA Technologies] and 89 μL of 5 X SSC (1 X SSC = 150 mM NaCl, 15 mM Sodium Citrate pH 7.0), heating to 95 °C for 10 min, cooling on ice for 30 s, then incubating for 5 min at room temperature. A 0.6-mL tube of Streptavidin MagneSphere Paramagnetic Particles (1 mg/mL) (Promega) was washed three times with 0.3 mL 5 X SSC. 100 μL of washed magnetic beads (1 mg/mL) was added to the DNA and incubated 15 min at room temperature, washed three times with 200 μL of 2 X SSC at room temperature, and washed three times with 200 μL of 1 X SSC at optimized temperature [70 °C for $(CA)_{15}$, 63 °C for $(CT)_{15}$, 60 °C for $(GATA)_6$] for 3 min. The DNA was eluted from the beads into 50 μL water by incubating for 5 min at 95 °C, washed

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Table 1 Characteristics of polymorphic microsatellites from *Anthonomus grandis* in a survey of 90 individuals collected in southern Texas and northeastern Mexico. H_O, observed heterozygosity. H_E, expected heterozygosity

	Primer sequences (5′–3′)	Repeat motif	No. of alleles	Size range (bp)	H_O	H_E	P*	GenBank accession no.
AG-D1	F: TGGTGAGGGGTGTGATATGAT R: GGAAGAACTAAAAACAAATGACGAA	$(GT)_9(GA)_2$	4	112–118	0.511	0.595	0.0041	AY611508
AG-D2	F: CATGGTTGGCCTACACCTTT R: GCCATCCGACAAATTCAATA	$(CG)_2(TG)_8$	3	123–127	0.500	0.595	0.1562	AY611509
AG-D3	F: CAAACGACGCTATTAGCTTGAA R: CGATAACACCCCGAAAATTG	$(CA)_{11}(GA)_2(CA)_2$	6	148–160	0.589	0.623	0.4724	AY611510
AG-D4	F: AGTTGCATATCGCGTCTCCT R: TCACTCTCCCAGTACATTTACCC	$(GT)_{10}$	7	206–220	0.544	0.587	0.0323	AY611511
AG-D5	F: AAGTTCATCGGTCGAGCAGT R: GCTGCGAAGGAATGAGAAAA	$(GT)_{10}$	10	243–265	0.656	0.737	0.0607	AY611512
AG-D6	F: GCGAGCGCTTCGTAGTTAAT R: GATCAAGTTCACATCACAAACA	$(GT)_4TT(GT)_8$	6	92–104	0.633	0.726	0.0314	AY611513
AG-D7	F: CAAAGGGTTGCGGATTTAAG R: CATTCGATACACCGCAACTG	$(GT)_{10}$	3	118–122	0.300	0.497	0.0003	AY611514
AG-D8	F: ATCATAAACGGGAACCACCA R: ATATCCCTCCGGAAGTGCTC	$(AG)_6$	3	132–136	0.089	0.383	0.0001	AY611515
AG-D9	F: TGTAATTTAATTATGATGGCAATTT R: ATCGATACTCCCCCTTAGGC	$(GT)_9(AT)_2$	5	132–142	0.344	0.614	0.0001	AY611516
AG-D10	F: CGGCTCGAGTTACACCGATA R: TTGGCACCGGATTTATTTTC	$(AG)_{12}$	9	174–198	0.556	0.647	0.0179	AY611517
AG-D11	F: TAAAACGTCGTTGGCCTCTC R: CAGGCCTTTCGATTTAATGC	$(GA)_{11}GC(GA)_2$	8	221–235	0.444	0.459	0.7768	AY611518
AG-D12	F: TGAGAGTGGAACGTGAGGTG R: CGAATTCCTTCATGGGCTTA	$(GA)_7AAGAAA(GA)_3$	4	290–296	0.311	0.461	0.0002	AY611519
AG-D13	F: GCTCAATGTCCCCCTCAAGT R: GTAGCAGCCCTTTTGCAGAC	$(GT)_{11}$	8	95–109	0.433	0.693	0.0001	AY611520
AG-D14	F: AATGAGGAAATGGGTCCACA R: AAACTGGTCCGTGGTAATCG	$(GA)_{13}$	6	130–145	0.689	0.720	0.0001	AY611521

*Hardy–Weinberg exact test, GENEPOP version 3.1b (Raymond & Rousset 1995).
In all cases, the forward primer was the one labelled for PCR.
Multiplexing: MP1, AG-D2 + AG-D5 + AG-D13; MP2, AG-D1 + AG-D3 + AG-D4; MP3, AG-D6 + AG-D9 + AG-D11; MP4, AG-D7 + AG-D10 + AG-D14; MP5, AG-D8 + AG-D12.

three times in a Microcon 100 spin-filter (Millipore), and concentrated to 15 μL. The DNA was reamplified by PCR with the EP-3 primer. PCR products were separated in a 2% LMP agarose gel in 1X TAE buffer, and the desired size fragment was purified.

The final PCR product was ligated into pGEM T-vector (Promega), and plasmid DNA was isolated using the QIAprep Spin Miniprep kit (Qiagen). Plasmid DNA was sequenced using a Beckman-Coulter CEQ 8000 Genetic Analysis System, and primers were designed based on sequences flanking repeat elements with the help of PRIMER3 software (Rozen & Skaletsky 2000). A total of 168 clones were sequenced, and 23 clones were finally chosen to design microsatellite primer sets. The 5′-end of forward PCR primers were labelled with Beckman-Coulter phosphoramidite flourophores enabling detection by the automated sequencer.

Preliminary screening of the 23 microsatellites that were amplified revealed that 14 were polymorphic (Table 1). Subsets of the 14 polymorphic microsatellites were tested for compatibility in PCR multiplexing. Reaction solutions consisted of 15 ng genomic DNA, 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.16 μM each primer, and 0.5 unit *Taq* polymerase in a total volume of 12.5 μL. The PCR conditions consisted of denaturation for 3 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 57 °C, and 1 min at 72 °C, and a final extension at 72 °C for 10 min. Multiplexing was successful for the subsets AG-D2 + AG-D5 + AG-D13, AG-D1 + AG-D3 + AG-D4, AG-D6 + AG-D9 + AG-D11, AG-D7 + AG-D10 + AG-D14, and AG-D8 + AG-D12.

Variability of the 14 microsatellite loci was surveyed in 90 boll weevil individuals collected in southern Texas and northeastern Mexico (Table 1). The number of alleles per locus ranged from three to 10, and heterozygosity values

ranged from 0.459 to 0.737. Observed heterozygosity was generally less than the expected, with significant deviations ($P < 0.05$) in 10 of 14 loci (Table 1). After correction for multiple tests (adjusted significance [5%] threshold = 0.00357), six markers still revealed deviation from Hardy–Weinberg equilibrium. Although the occurrence of null alleles cannot be excluded in these markers, a preliminary pedigree analysis indicated that 12 of the microsatellites are inherited in a Mendelian fashion (data not shown) and appear to be promising for population genetics studies of the boll weevil. However, AG-D13 and AG-D14 are not likely to follow a Mendelian mode of inheritance and their primers will need to be redesigned.

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